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Ultrastructural study of neuromuscular junction in rectus femoris muscle of streptozotocin-diabetic rats

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Summary. The neuromuscular junctions (NMJ) from rectus femoris muscle in streptozotocin (STZ) -induced diabetic rats were examined by electron microscopy eight weeks after the STZ injection. When compared to controls and vehicle-injected groups, both the axon terminal and the junctional sarcoplasm showed serious alterations including mitochondrial degeneration, presence of myeloid bodies, breakdown of presynaptic membrane and changes in the form of the synaptic vesicles. The results suggest that NMJ can contribute to the pathogenesis of diabetic proximal myopathy.

Key words: Quadriceps muscle, Rat, Streptozotocin diabetes, Ultrastructure, Neuromuscular junction

Introduction

The syndrome of diabetic symmetrical proximal motor neuropathy, also called «diabetic amyotrophy» has been described as a disease entity related to diabetic peripheral neuropathy (Brown and Asbury, 1984). Clinical criteria of this syndrome include wasting and weakness of pelvifemoral muscles without sensory impairment. This disorder is generally painful and it is often asymmetrical at first, but it may become symmetrical as it progresses (Picard and De la Monte, 1987). Patients with diabetes frequently complain of muscular weakness which has been thought to be due to metabolic alterations of the muscle resulting from the disease (Fecko and Klueber, 1988).

Despite the extensive literature concerning the neuropathy related to diabetes, only restricted information describes changes in the associated muscle (Fecko and Klueber, 1988; Klueber et al., 1989) and the pathological basis for proximal motor neuropathy in diabetes is not known yet. The changes described in the neuromuscular junction (NMJ) may play an important role in the pathogenesis of diabetic myopathy as has been previously postulated (Bestetti et al., 1971; Chokroverty et al., 1977, 1988; Chokroverty, 1982; Pachter, 1986; Fecko and Klueber, 1988). Light microscopy and histochemical methods have demonstrated "hyperneurotization" (Hildebrand et al., 1986). Also, reduction of myo-neural contact (Woolf and Manlis, 1987) and degeneration and neoformation of motor end plates (Awad and Kottke, 1977) have been found.

Ultrastructural studies performed in experimental diabetes from extraocular muscles from the diabetic mouse model (Pachter, 1986), rat diaphragm (Bestetti et al., 1971), and in extensor digitorum longus from a genetic model of diabetes in mice (Fecko and Klueber, 1988; Chokroverty et al., 1988) have shown significant signs of degeneration, denervation and abnormal structure of NMJs.

Animal models of diabetes can develop peripheral neuropathy (Sharma and Thomas, 1987) which are useful to study the physiological and pathological changes produced in muscle by experimental diabetes (Challiss et al., 1989).

In the present study we have chosen the rectus femoris muscle (RFM) of adult male rats 8 weeks after the induction of streptozotocin (STZ) diabetes. This muscle is a good model for studying diabetic proximal myopathy since it is a pelvi-femoral girdle muscle. Also, histochemical analysis after 8 weeks of disease is available (Medina-Sánchez et al., 1991) and to our knowledge it has not been previously studied in experimental diabetic myopathy.

Materials and methods

Animals

Twenty adult male Wistar rats ranging from 230 to 280 g body weight, (13-18 weeks old) were injected with STZ (ZANOSAR) solution (60 mg/500 µl/kg body weight adjusted to pH 4.5 with 50 mM citrate buffer), into the tail vein to induce experimental diabetes mellitus. All rats were individually housed in cages under a light:dark cycle of 12:12 hours the lights being automatically turned on at 07:00 a.m. The temperature was controlled (20±2° C) and the rats were given free access to water and PANLAB, S.L. rat chow (following the Institutional guide for the care and use of laboratory

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animals).

The diabetic (N=7), vehicle injected (N=7) and control (N=6) groups were killed 8 weeks after STZ injection. At the time of sacrifice, blood sugar was determined using a routine glucose oxidase method. Blood sugar levels above 250 mg/dl were considered pathologic.

Ultrastructural study

Under chloral hydrate anaesthesia (350 mg/kg/body weight), all the animals were perfused through the left ventricle with 2% glutaraldehyde, 1.5% paraformaldehyde solution, phosphate-buffered to pH 7.4 for 15 minutes. Then the RFM was removed. Following removal, it was left in fresh fixative solution for at least an additional 48 h. Samples were postfixed in 1% osmium tetroxide buffered with 0.2 M sodium phosphate (pH 7.4), dehydrated in graded acetones and embedded in EPON 812. Semithin resin sections (1 μm), were stained with toluidine blue and thin sections were taken through the midbelly region of the muscle, stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109, electron microscope.

Morphometric study

A morphometric analysis of synaptic vesicles in nerve endings of myoneural synapsis from intact, vehicle injected and experimental animals was performed using a semiautomatic image analysis system (Videoplan, Kontron, FRG) according to the methodology described by Vega et al. (1988). The synaptic vesicles in 26 axonal endings from controls, 30 axonal endings from vehicle-injected rats and 29 axonal endings from STZ-injected rats were studied. In microphotographs with a final magnification of x 32000 the following parameters were evaluated: a) diameter of the vesicle; and b) circular factor of form (CFF). The more irregular a structure is, the more the CFF value deviated from 1.

Statistical analysis

Statistical analysis of glycemia and morphometric results was performed by analysis of variance (ANOVA) followed by a Student-Newman-Keuls test. All data are expressed as mean ± S.E. and a p value <0.05 was considered significant.

Results

Physical data

The blood sugars in the STZ-injected rats were significantly higher than the other two groups (Fig. 1).

Morphological data

The ultrastructural analysis of neuromuscular junction from intact and vehicle-injected animals showed its typical morphological characteristics. The terminal axon displayed a variable number of mitochondria and synaptic vesicles (Fig. 2). Mitochondria were irregular in shape and displayed well-developed cristae. The synaptic vesicles were distributed throughout the axoplasm, mainly located close to the junctional folds. The morphometric analysis of synaptic vesicles is shown in Table 1. The muscle sole plates in the vehicle-injected animals showed no alterations.

All the STZ-diabetic animals showed changes in the NMJs, both in the axon terminal and in the motor end plate. In the axonal ending, mitochondrial degeneration changes were seen as swollen mitochondria, disorganization of the cristae, and breakdown of the

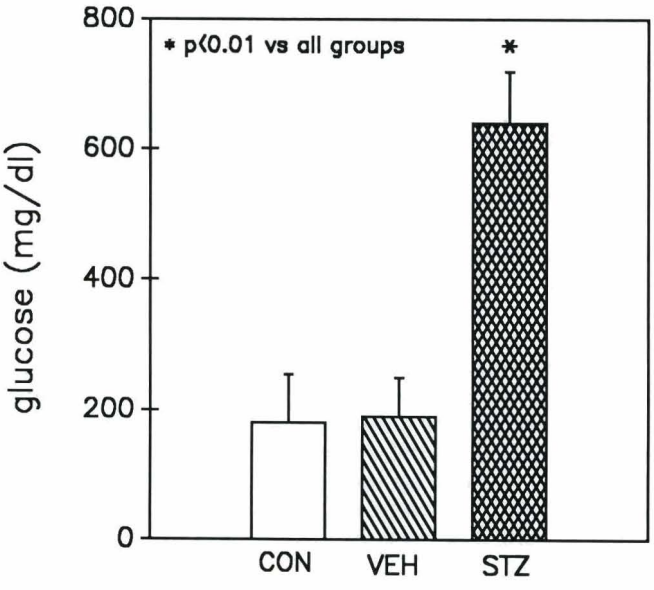


Fig. 1. Final blood glucose levels from intact (CONT), vehicle-injected (VEH) and experimental (STZ) groups.

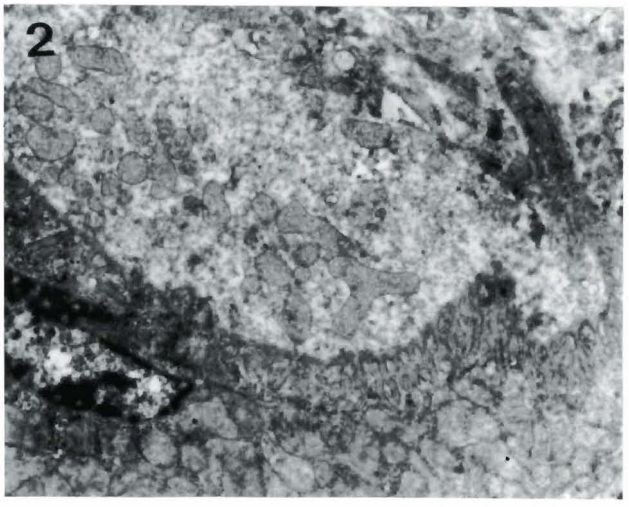


Fig. 2. Normal myoneural junction from control rectus femoris muscle. x 9,000

mitochondria were also observed (Figs. 3, 4). In addition, some electron-dense bodies and a quantity of myelin-like debris could also be observed (Figs. 3, 5). Examination of some of the myoneural junctions exhibited breakdown of the presynaptic membrane (Fig. 4).

Although a slight increase in mean diameter of synaptic vesicles in diabetic animals was observed (Table 1), no significant differences were found.

The analysis of CFF revealed that the vesicles found in the diabetic NMJs, were more irregular (less similar to the circle) than those of intact and vehicle-injected animals.

The muscle sole plate in the different diabetic animals was not uniformly affected. However, most of the diabetic animals exhibited various types of alterations, including mitochondrial degeneration, disorientation of t-tubular system, disruption of myofibrils and myofilaments and finally an increment in the number of lipid-like droplets and glycogen granules. In addition, myelin-like structures were observed in neighbouring regions (Fig. 4).

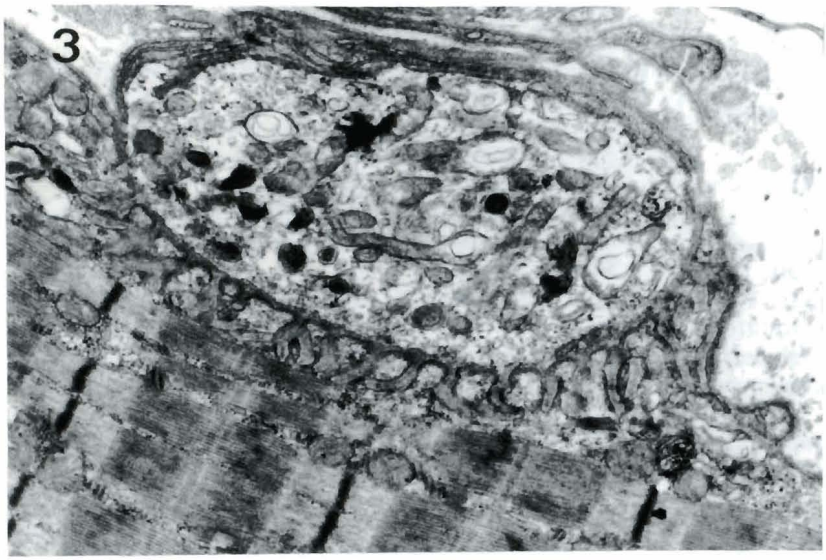
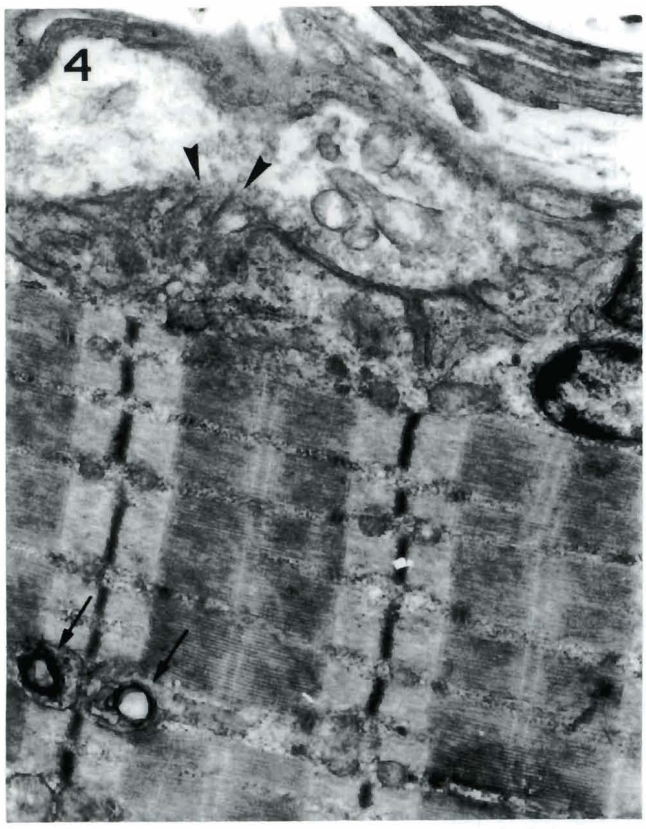


Fig. 3. Electron micrograph from an STZ- diabetic NMJ. This junction exhibits degeneration within the axonal ending. Note the electron-dense bodies and the mitochondrial degeneration. x 14,500

Discussion

It is well known that three major neuropathic syndromes occur in diabetes (Brown and Asbury, 1984). The most common is a distal symmetrical polyneuropathy. Less frequent are proximal motor neuropathies and focal and multifocal neuropathies. Symmetrical proximal motor neuropathy ("diabetic amyotrophy"), affects mainly the quadriceps muscle. Both diffuse metabolic



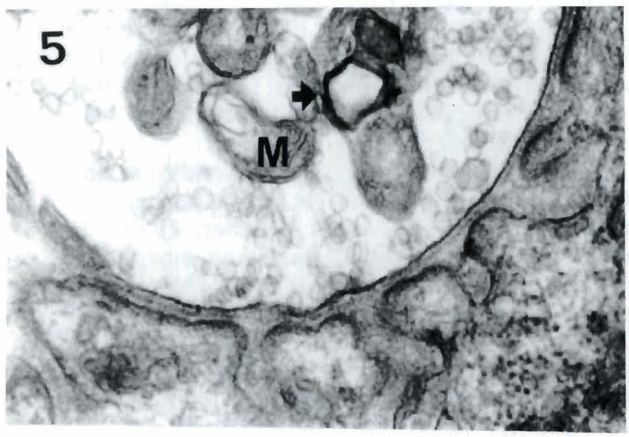


Fig. 5. Degenerated mitochondria (M) and myelin figures (arrow) in an NMJ from STZ- diabetic muscle. x 28,000

Fig. 4. Electron micrograph of an STZ-diabetic NMJ. Note the breakdown of mitochondria and the dysruption of the presynaptic membrane (arrowheads). Two myelin bodies can be observed in the myofibre below the end plate (arrows). x 20,000

Table 1. Morphometric analysis of presynaptic vesicles in motor end plates of rectus femoris muscles from control, vehicle injected and STZ-induced diabetic rats. The values are expressed as means ± SEM.

	Circle diameter (nm)	Circular factor of form
Control (n=26)	519±68	0.88±0.02
Vehicle injected (n=30)	530±86	0.86±0.02
Streptozotocin (n=29)	603±113	0.69±0.09*

* $p<0.05$ vs. control and vehicle injected groups. n: number of analyzed motor end plates.

and vascular abnormalities have been implicated as a possible cause (Asbury, 1987).

Diabetic proximal motor neuropathy affects muscles which are innervated by femoral, obturator, sciatic and gluteal nerves as well as lumbar spinal rami. This distribution could result from a process located anywhere from anterior horn cells to intramuscular nerve endings. The site of nerve involvement is not known yet (Asbury, 1987).

In our study, both the axon terminal and junctional sarcoplasm from rectus femoris NMJ of STZ-diabetic rats showed serious alterations.

Similar findings have been previously described in human diabetic proximal muscle (Chokroverty, 1982) and in distal muscles from a genetic model of diabetic animal (Fecko and Klueber, 1988; Chokroverty et al., 1988). Although inclusion bodies in the sole plate nucleus have been reported (Pachter, 1986) in oculomotor muscles from STZ-diabetic rats, they were not observed in our study.

Disruption of the presynaptic membrane has also been reported in a distal muscle from a genetic model of murine diabetes (Fecko and Klueber, 1988). To our knowledge, changes in the size and morphology of synaptic vesicles during diabetes, have not been previously reported.

Most of the findings shown in the motor endplates in the present study are recognized as signs of nerve degeneration (Magnaini and Friedrich, 1981) and consequently result in a denervation and loss or reduction in muscle function. Thus we think that, at least in part, the proximal muscle weakness present in the course of diabetes can be attributed to the changes in the neuromuscular junction, as has been previously postulated (Bestetti et al., 1971; Klueber et al., 1989). The degeneration of the axonal ending in myoneural synapsis and subsequently «muscle -trophic factors» could be responsible for the diabetic myopathy, since the muscular changes are similar to those reported after denervation myopathy. Indeed, neuropathic factors are possibly involved in the pathogenesis of diabetic proximal motor neuropathy. Further studies, including diabetic patients and animal models are needed in order to improve our knowledge of the diabetic amyotrophy.

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